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Protoplast isolation and culture from *Kalanchoë* species: optimization of plant growth regulator concentration for efficient callus production

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Abstract

A high yield of isolated protoplasts and efficient regeneration protocols are prerequisites for successful development of somatic hybrids. In the present study, protoplast isolation and regeneration were evaluated in 12 *Kalanchoë* accessions belonging to nine species. The highest protoplast yield was obtained from *K. blossfeldiana* ‘Charming Red Meadow’ with $10.78 \pm 0.51 \times 10^5$ protoplasts per gram fresh weight. We observed significant differences of protoplast yield while there was no distinct difference in viability among the accessions. Seven accessions reached the microcolony stage and four developed microcalli in medium supplemented with 1.0 mg/l 1-naphthaleneacetic acid (NAA), 0.5 mg/l 6-benzylaminopurine (BAP) and 0.5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D). Using five selected accessions we optimized the PGR (plant growth regulators) concentrations using combinations of NAA, BAP and 2,4-D. *K. blossfeldiana* cultivars ‘Charming Red Meadow’ and ‘Paris’ produced significantly different numbers of calli depending on the PGR concentrations. For plant regeneration, the medium was supplemented with 1 mg/l NAA and 2 mg/l BAP or 2 mg/l zeatin. Shoots were regenerated on medium supplemented with NAA and BAP for *K. blossfeldiana* ‘Charming Red Meadow’ and *K. blossfeldiana* ‘Paris’. The plants successfully developed roots on the medium supplemented with IAA. The medium containing zeatin induced root formation directly from callus in *K. blossfeldiana* ‘Charming Red Meadow’. Our findings have the potential to facilitate the use of *Kalanchoë* species in somatic hybridization breeding programs.

Key Message

The study revealed a strong genotype-dependent efficiency of colony and microcallus formation. Plants were regenerated from two *Kalanchoë blossfeldiana* cultivars on medium supplemented with NAA and BAP.

Keywords Organogenesis · Plant growth regulators · Plant regeneration · Protoplast-derived callus · Protoplast isolation

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Abbreviations

2,4-D	2,4-Dichlorophenoxy acetic acid
BAP	6-Benzylaminopurine
FDA	Fluorescein diacetate
gfw	Gram fresh weight
IAA	Indole-3-acetic acid
MES	2-(N-Morpholino) ethane sulfonic acid
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
PGR	Plant growth regulators

Introduction

Kalanchoë is a widespread flowering plant genus belonging to the Crassulaceae family. It comprises around 140 species that are mainly succulent plants native to Madagascar

and East and South Africa (Descouings 2003). *Kalanchoë blossfeldiana* is the most exploited species of the genus and it gave rise to the majority of commercial cultivars. *Kalanchoë* cultivars are widely used as potted plants due to plentiful flowering, longevity of individual flowers, postharvest performance, and low-care requirements (Mackenzie et al. 2018). *Kalanchoë* is the second most economically important potted plant in Europe with a turnover of 69 million euros in 2017 (Royal Flora Holland 2018). The genus is also increasingly utilized as cut flowers (Mackenzie et al. 2018). Breeding of new cultivars started as early as 1930s aiming at the development of compact cultivars with various flower colours. Currently, the cultivar improvement is achieved using cross-breeding and interspecific hybridization (Izumikawa et al. 2008; Kuligowska et al. 2015a), though genetic engineering has also been reported (Lütken et al. 2011). The expansion of the gene pools and generation of new *Kalanchoë* cultivars using genetic resources of wild species is of great interest (Mackenzie et al. 2018). Successful interspecific hybridization was accomplished within the *Kalanchoë* genus (Izumikawa et al. 2008; Kuligowska et al. 2015a; Huang and Chu 2017), however, development of hybrids is limited due to hybridization barriers related to sexual reproduction (Kuligowska et al. 2015b). Fusion of protoplasts from different species provides a practical breeding tool that can bypass sexual hybridization-related barriers. Nevertheless, development of an efficient protoplast-to-plant system is often a limiting step in somatic hybridization (Eeckhaut et al. 2013). Protoplast regeneration can be influenced by a number of factors, including donor plant material and genotype, culture system and media composition. In particular, the concentration and ratios of main plant growth regulators, auxins and cytokinins are of key importance (Davey et al. 2005). Thus, to obtain a high regeneration frequency, the protoplast culture conditions need to be optimized.

Previous studies of *Kalanchoë* in vitro cultures documented shoot regeneration using different explants and hormone combinations. Varga et al. 1988 examined the effect of auxins and cytokinins in *K. blossfeldiana* (indole-3-acetic acid—IAA, indole-3-butyric acid—IBA, zetin, kinetin and N6-(2-Isopentenyl)adenine—2iP) on shoot regeneration from leaf-derived callus. Khan et al. (2006) evaluated effects of BAP, NAA and PGR-free medium on direct regeneration of *Kalanchoë tomentosa* from shoot tips. The successful shoot regeneration from leaf and internode explants using thidiazuron (TDZ) was also reported in *K. blossfeldiana* (Sanikhani et al. 2006; Lütken et al. 2011). Furthermore, one study reported regeneration of plants from protoplasts of a single *K. blossfeldiana* cultivar (Castelblanque et al. 2010). Electric fusion was reported between isolated vacuoles and protoplasts of *K. daigremontiana* (Vienken et al. 1981), while neither chemical fusion nor successful somatic hybridization was obtained in *Kalanchoë* genus.

In the present study we present a modified protocol for isolation of mesophyll protoplasts from nine *Kalanchoë* species. The results reveal the regeneration ability of several *Kalanchoë* species and the role of plant growth regulators in protoplast regeneration.

Materials and methods

Plant material

Nine *Kalanchoë* species were used in the present experiments: *K. aromatica*, three *K. blossfeldiana* cultivars: ‘Charming Red Meadow’, ‘Charlie’ and ‘Paris’, *K. gracilipes*, *K. marnieriana*, *K. miniata*, *K. pinnata* wild type (WT) and a *root oncogenic loci (rol)*—gene transformed type (unpublished), *K. pumila*, *K. streptantha* and *K. rotundifolia*. The plant cuttings were established in peat soil (NPK 14-7-15 0.650 kg per m³ + micro 50 g per m³, soil type: ‘Pindstrup Substrate No. 1’, Pindstrup Mosebrug A/S, Ryomgård, Denmark) in 11 cm diameter pots and grown in a greenhouse with a day/night temperature of 24 ± 4 °C/18 ± 4 °C and a photoperiod of 16 h. Additional illumination of 260 µmol s⁻¹ m⁻² supplied by Philips Master SON-T PIA Green Power 400 W, was provided when natural light irradiance was below 180 µmol s⁻¹ m⁻². Ebb-flow irrigation was performed with water containing fertilizer (Pioneer NPK Macro 14-3-23 + Mg combined with Pioneer Micro; pH 5.5; EC = 1.3, Azelis, Antwerp, Belgium) once a week.

Axenic plant cultures

Stem segments with axillary buds were used as in vitro explant source. The plant stems were collected from 1- to 2-month-old plants and surface sterilized according to Lütken et al. (2011). Explants of 3 ± 0.2 cm were cultured on MS medium (Murashige and Skoog 1962) containing 30 g/l sucrose, 0.5 g/l 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.66 g/l CaCl₂, 0.08 g/l Ethylenediamino-*N,N'*-bis(2-hydroxy-phenyl)acetic acid iron (EDDHA-Fe), 1 ml/l Atamon™ (sodium benzoate), 100 mg/l timentin, 0.05 mg/l IAA and 3 g/l gelrite. The pH was adjusted to 5.8 prior to autoclaving. All cultures were maintained in a growth chamber at 24 °C with a 16 h photoperiod and a light intensity of 150 µmol s⁻¹ m⁻². Plants were subcultured monthly/every second month depending on the cultivar, and maintained as a source of leaf material for protoplast isolation.

Protoplast isolation

Protoplasts were isolated from young fully expanded leaves of in vitro plants. One gram of leaf tissue was cut into 1–2 mm stripes in 10 ml of washing medium (WM: 1.65 g/l

MS macro elements, 106 g/l mannitol, 7 g/l glycine, 1.5 g/l CaCl_2 , 0.5 g/l MES, pH 5.8, autoclaved) in 5 cm Petri dishes. After 30 min incubation, the WM solution was replaced with filter-sterilized (0.2 μm , Whatman, International Ltd., Maidstone, Kent, UK) enzymatic solution containing 0.5% (w/v) Cellulase Onozuka R-10 (Duchefa Biochemie, Haarlem, The Netherlands) and 0.1% (w/v) Driselase (Sigma-Aldrich, St. Louis, US) dissolved in WM. Digestion was performed at room temperature in darkness with shaking at 40 rpm for 16–18 h. The protoplast suspension was filtered through 4-layer sterile cheesecloth and centrifuged at $100\times g$ for 10 min. The protoplast pellet was suspended in 10 ml flotation medium (FM: 205 g/l sucrose, 0.64 g/l MES, pH 6.0, filter-sterilized). Protoplasts were purified by density gradient centrifugation at $50\times g$ for 10 min, after layering 1 ml of modified W5 (Menczel et al. 1981) (0.46 g/l sucrose, 18.38 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.37 g/l KCl, 9.076 g/l NaCl, pH 5.8, autoclaved) on the FM. The collected protoplasts were suspended in 10 ml W5 and centrifuged at $100\times g$ for 10 min to collect protoplasts.

Yield and viability assessment

The purified protoplasts were counted using a Fuchs-Rosenthal haemocytometer chamber. The yield was expressed as number of protoplasts per gram fresh weight (gfw). The protoplast viability was determined by fluorescein diacetate (FDA) staining according to Widholm (1972). For viability assessment, 0.5 μl FDA solution (25 mg FDA dissolved in 5 ml acetone, Sigma-Aldrich, St. Louis, USA) was added to 100 μl protoplast suspension. Protoplasts were observed under a fluorescence microscope (Leica DM2000 LED, Leica, Germany; excitation filter BP 450–490 nm, dichromatic mirror: 510 nm and emission filter: LP 515 nm) equipped with a digital camera (Leica DFC420, Leica, Germany). The protoplast viability was determined as the ratio between the green fluorescent protoplasts and the total number of observed protoplasts. The mean protoplast yield and viability were calculated based on two independent isolations per genotype. At least three microscopic fields per isolation with minimum 50 protoplasts were screened.

Protoplast culture

Protoplasts were suspended in culture medium 1 (M1—Table 1) and cultured at a concentration of 10^5 protoplasts ml^{-1} in 1 ml or 2.5 ml depending on the experiment. Cultures were refreshed weekly by adding 0.4 volume of fresh medium with lower mannitol concentration (M2—Table 1). After 4 weeks, the culture medium was replaced by the same volume of medium 3 (M3—Table 1). In M3, the cultures were first cultured for 2 weeks in dark, followed by 2 weeks of 16 h photoperiod. Cultures were inspected regularly under

a microscope (Leica DM2000 LED, Leica, Germany) for cell division and microcolony formation. The number of microcalli (1–2 mm diameter) was determined and they were transferred using tweezers to 5 cm Petri dishes containing solid medium 4 (M4—Table 1). The developed shoots were separated from organogenic callus and cultured on solid medium 5 (M5—Table 1). The media composition, culture conditions and culture duration are shown in Table 1. PGR concentrations differed between experiment 1 and 2 and are shown in Table 2 and 3.

Experiment 1: Protoplast yield and viability screening

Nine *Kalanchoë* species (12 accessions) described above were used. The protoplast isolation, yield and viability assessment were performed as described above using 0.5 g leaf material per isolation. The protoplast cultures were established with 2.5 ml of culture per 5 cm Petri dish. The media were as described in Table 1 and they were supplemented with PGR according to Table 2. The protoplast cultures were observed daily during the first week and once a week during the remaining culture period. Three independent isolations were carried out for each plant accession. The mean values and standard deviations were calculated. Analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) tests were performed using SPSS 9.1 (SPSS Institute, Chicago, USA) to assess the effect of the treatments.

Experiment 2: Optimization of PGR concentrations

To determine the effect of plant PGR on protoplast regeneration, protoplast cultures with 12 different PGR concentrations and one control (without PGR) were established using five *Kalanchoë* accessions: *K. blossfeldiana* 'Charming Red Meadow', 'Charlie' and 'Paris', *K. marnieriana* and *K. pinnata* WT. The protocols for protoplast isolation and yield assessment were as described above. The quantity of leaf material for protoplast isolation was determined based on the result of experiment 1 to yield 39×10^5 protoplasts. The protoplast cultures were set up in 35 mm Petri dishes with 1 ml of protoplast culture at a density of 10^5 protoplasts ml^{-1} . The cultures were performed under the conditions described in Table 1. The media were supplemented with PGR concentrations according to Table 3. Each treatment consisted of 3 technical replicates (Petri dish) and was repeated twice. After 5 weeks in solid medium M4, if no shoot or root formation was observed, the calli were maintained on M4 for an additional 4 weeks. Analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) tests were performed using SPSS 9.1 (SPSS Institute, Chicago, USA) to assess the effect of the treatments.

Table 1 Media used for the culture of *Kalanchoë* protoplasts, callus proliferation, and plant regeneration

Medium	Explant	Composition	Type	Duration	Conditions
M1	Protoplasts	Kao and Michayluk (1975) macro- and micronutrients, 40 g/l sucrose, 5 g/l myo-inositol, 3 g/l xylitol, 0.5 g/l MES, 0.05 g/l ascorbic acid, 0.01 g/l adenine hemisulfate, Schenk and Hildebrandt (1972) vitamins, 50 g/l mannitol, 100 mg/l timentin, pH 5.8	Liquid	1 week	Darkness, 24 ± 1 °C
M2	Microcolonies	Kao and Michayluk (1975) macro- and micronutrients, 40 g/l sucrose, 5 g/l myo-inositol, 3 g/l xylitol, 0.5 g/l MES, 0.05 g/l ascorbic acid, 0.01 g/l adenine hemisulfate, Schenk and Hildebrandt (1972) vitamins, 42 g/l mannitol, 100 mg/l timentin, pH 5.8	Liquid	3 weeks	Darkness, 24 ± 1 °C
M3	Microcalli	Murashige and Skoog (1962) macro- and micronutrients and vitamins, 30 g/l sucrose, 0.1 g/l myo-inositol, 0.5 g/l MES, 35 g/l mannitol, 100 mg/l timentin, pH 5.8	Liquid	2 weeks + 2 weeks	Darkness, 24 ± 1 °C; 16 h photoperiod, 150 µmol s ⁻¹ m ⁻² light intensity, 24 ± 1 °C/15 ± 1 °C day/night
M4	Calli	Murashige and Skoog (1962) macro- and micronutrients and vitamins, 30 g/l sucrose, 0.1 g/l myo-inositol, 0.5 g/l MES, 35 g/l mannitol, 100 mg/l timentin, 3 g/l gelrite, pH 5.8	Solid	5 weeks (+ 4 weeks)	16 h photoperiod, 150 µmol s ⁻¹ m ⁻² light intensity, 24 ± 1 °C/15 ± 1 °C day/night
M5	Shoots	Murashige and Skoog (1962) macro- and micronutrients and vitamins, 30 g/l sucrose, 0.5 g/l MES, 100 mg/l timentin, 3 g/l gelrite, 1 ml/l Atamon™, pH 5.8	Solid	4 weeks	16 h photoperiod, 150 µmol s ⁻¹ m ⁻² light intensity, 24 ± 1 °C/15 ± 1 °C day/night

Table 2 Media PGR supplementations in experiment 1

Medium	PGR supplementation
M1	0.5 mg/l 2,4-D, 1.0 mg/l NAA, 0.5 mg/l BAP
M2	0.5 mg/l 2,4-D, 1.0 mg/l NAA, 0.5 mg/l BAP
M3	1.0 mg/l NAA, 2.0 mg/l BAP
M4	1.0 mg/l NAA, 2.0 mg/l BAP
	1.0 mg/l NAA, 2.0 mg/l zeatin

For details about media compositions see Table 1

2,4-D 2,4 dichlorophenoxy acetic acid, NAA: 1-naphthaleneacetic acid, BAP 6-benzylaminopurine

Results

Experiment 1: Protoplast yield and viability screening

The leaves of in vitro plants were used for protoplast isolation when they had developed two new leaf pairs. The time required to reach this developmental stage was between 4 and 6 weeks depending on the accessions (Supplementary Fig. 1). Viable protoplasts were isolated from all 12 accessions. They were spherical, with a diameter between 10

Table 3 Media PGR supplementations in experiment 2

Medium	PGR supplementation			
	Treatment	2,4-D (mg/l)	NAA (mg/l)	BAP (mg/l)
M1	control	0	0	0
	A	0.05	0.1	0.05
	B	0.1	0.1	0.05
	C	0.5	0.1	0.05
	D	1.0	0.1	0.05
	E	0.05	0.1	0.1
	F	0.1	0.1	0.1
	G	0.5	0.1	0.1
	H	1.0	0.1	0.1
	I	0.05	0.1	0.5
	J	0.1	0.1	0.5
	K	0.5	0.1	0.5
	L	1.0	0.1	0.5
M2	PGR treatments as in M1 (A to L)			
M3	PGR treatments as in M1 (A to L) without 2,4-D			
M4	1.0 mg/l NAA, 2.0 mg/l BAP			
	1.0 mg/l NAA, 2.0 mg/l zeatin			
M5	0.1 mg/l IAA			

For details about media compositions see Table 1

2,4-D 2,4 dichlorophenoxy acetic acid, NAA 1-naphthaleneacetic acid, BAP 6-benzylaminopurine

and 70 μm . Different protoplast types were observed following isolation (Fig. 1a-I–IV). The protoplast yield varied significantly among examined plants. The lowest yield was obtained for *K. rotundifolia* ($0.52 \pm 0.09 \times 10^5$ protoplasts per gfw), while the highest yield was obtained for *K. blossfeldiana* ‘Charming Red Meadow’ ($10.78 \pm 0.51 \times 10^5$ protoplasts per gfw) (Fig. 2). The protoplast viability did not differ significantly among accessions (Fig. 2).

Protoplasts of all accessions regenerated cell walls in vitro within 24 h. The first cell divisions (Table 4) were observed from 3 days to 7 days in all accessions. Microcolonies developed after 2–4 weeks from protoplasts of all *K. blossfeldiana* cultivars, *K. marnieriana*, *K. pinnata* WT, *K. rotundifolia* and *K. streptantha* (Table 4). After 8 weeks of culture, visible microcalli of 1–2 mm were obtained from

K. blossfeldiana ‘Charming Red Meadow’ and ‘Paris’, *K. pinnata* WT and *K. rotundifolia* (Table 4). *K. rotundifolia* had the highest calli yield, followed by *K. blossfeldiana* ‘Charming Red Meadow’, *K. pinnata* WT and *K. blossfeldiana* ‘Paris’ (Table 4). The calli were further transferred to the solid medium (Table 1 and 2). However, after 3 weeks they showed no growth, thus the culture was terminated.

Experiment 2: Optimization of PGR concentrations

Based on the results from experiment 1, the plants with high protoplast yield: *K. blossfeldiana* ‘Charming Red Meadow’, ‘Paris’, ‘Charlie’, *K. pinnata* WT and *K. marnieriana* were included in the optimization of culture conditions for regeneration. In experiment 2, the highest yield was observed for

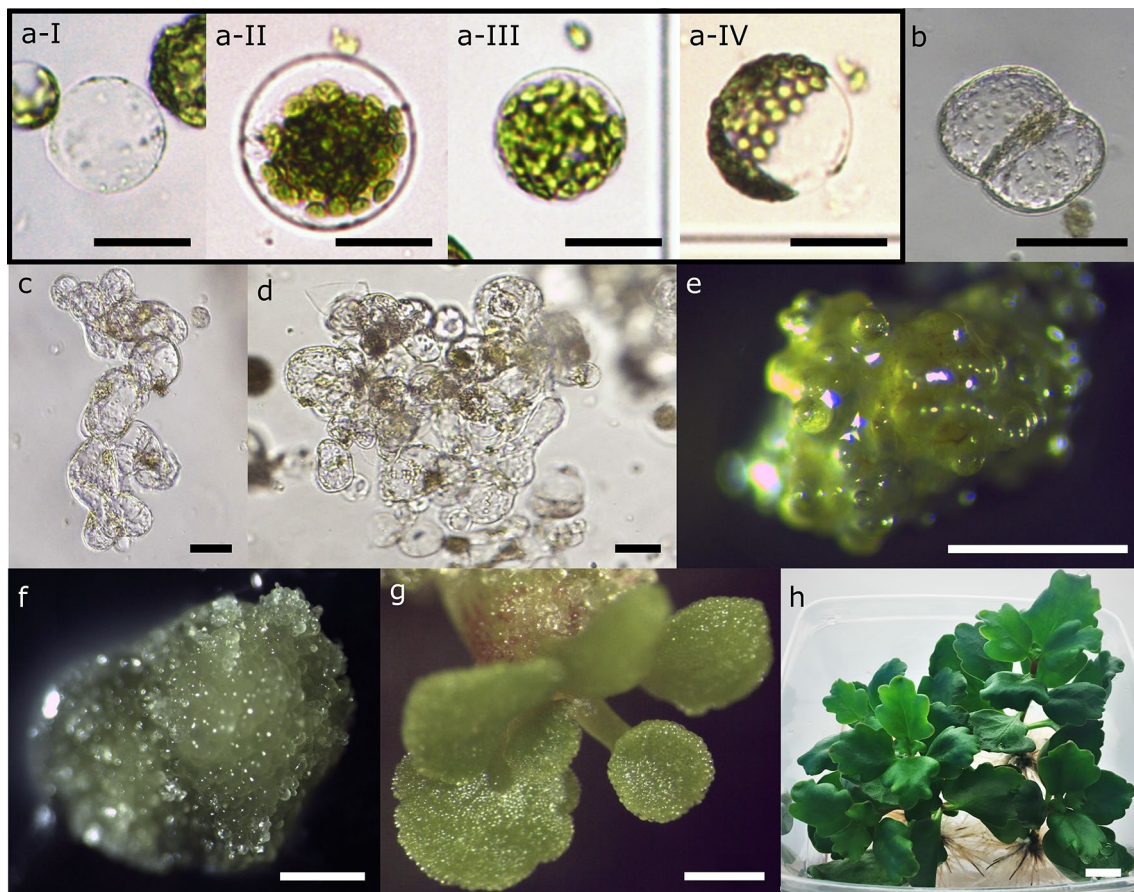


Fig. 1 Plant regeneration from protoplasts of *K. blossfeldiana* ‘Charming Red Meadow’ **a** different types of isolated protoplast **a**-I: small protoplast without chloroplasts; **a**-II: dense protoplast with chloroplasts concentrated in the middle of the cell; **a**-III: dense protoplast with chloroplasts distributed homogenously throughout the cytoplasm; **a**-IV: vacuolated protoplast with chloroplasts concentrated

in one zone of the cell; **b** protoplast division at 3 days after culture initiation; **c** microcolonies 2 weeks after culture initiation; **d** microcolonies 4 weeks after culture initiation; **e** green microcallus 8 weeks after culture initiation; **f** yellow microcallus 8 weeks after culture initiation; **g** shoot regenerated on the callus at 17 weeks; **h** regenerated plants at 22 weeks; scale bars: **a**, **b** 25 μm ; **c**, **d** 50 μm ; **e**–**h** 1 mm

K. pinnata WT at $7.57 \pm 0.86 \times 10^5$ protoplasts per gfw. *K. marnieriana* had the lowest yield at $2.56 \pm 0.60 \times 10^5$ protoplasts per gfw (Fig. 3).

The culture medium in experiment 2 was supplemented with a tenfold lower NAA concentration than in experiment 1, and different concentrations of BAP and 2, 4-D (Table 3). All protoplasts cultured on medium without PGR died within 3 days. In the cultures supplemented with PGR, protoplasts of *K. pinnata* WT and *K. marnieriana* rebuilt a cell wall and underwent first divisions, but did not develop

further. *K. blossfeldiana* ‘Charlie’ protoplasts formed microcolonies, but they stopped their development in the fifth week of culture after transfer to 16 h photoperiod. *K. blossfeldiana* ‘Charming Red Meadow’ and ‘Paris’ protoplasts exhibited stable mitosis and developed microcalli that were transferred to solid medium after 8 weeks (Fig. 1b–f). The *K. blossfeldiana* ‘Charming Red Meadow’ calli obtained in both repetitions of experiment 2 followed a similar pattern. The lowest number of calli was obtained in media supplemented with 0.05 mg/l BAP, and the highest number of calli

Fig. 2 Protoplast yield and viability of 12 *Kalanchoë* accessions in experiment 1; bars represent mean \pm SD, $n=3$; yield values followed by different letters are significantly different ($p \leq 0.05$) according to Tukey’s honestly significant difference test; gfw: gram fresh weight; *K. b.*: *K. blossfeldiana*; ‘Ch. R. Meadow’: ‘Charming Red Meadow’

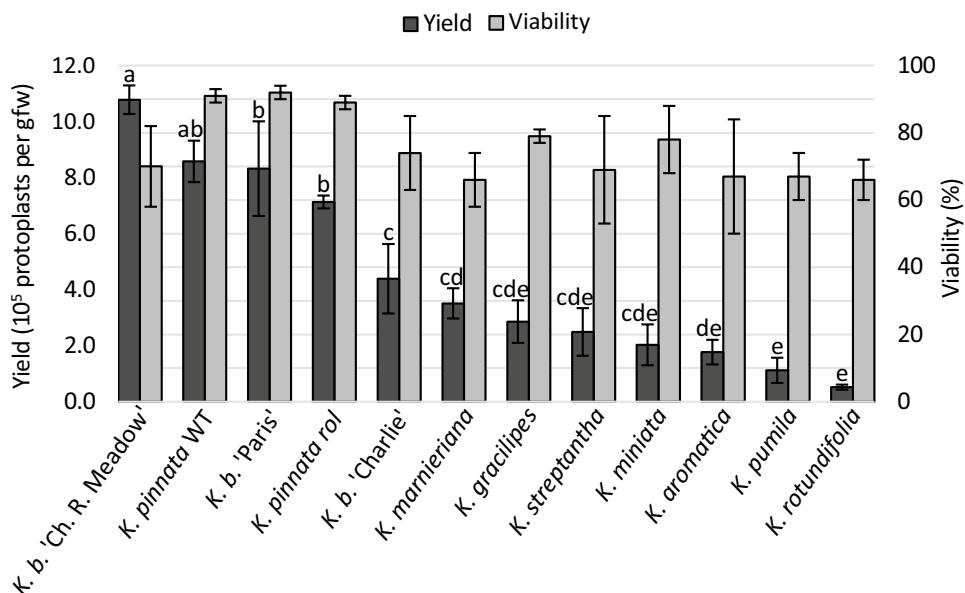


Table 4 Protoplast regeneration status from different *Kalanchoë* accessions in experiment 1

Plant name ¹	First division ²	Microcolonies ^{2,3}	Microcalli ^{2,3}	Number of calli per 10^5 protoplasts
<i>K. aromatica</i>	1 week	–	–	
<i>K. b.</i> 'Charlie'	1 week	4 weeks	–	
<i>K. b.</i> 'Charming Red Meadow'	3 days	2 weeks	8 weeks	46.8 ± 3.6 a
<i>K. b.</i> 'Paris'	3 days	2 weeks	8 weeks	11.33 ± 1.8 b
<i>K. gracilipes</i>	1 week	–	–	
<i>K. marnieriana</i>	1 week	4 weeks	–	
<i>K. miniata</i>	1 week	–	–	
<i>K. pinnata</i> WT	3 days	2 weeks	8 weeks	44.3 ± 2.7 a
<i>K. pinnata</i> rol	3 days	–	–	
<i>K. pumila</i>	1 week	–	–	
<i>K. rotundifolia</i>	3 days	3 weeks	8 weeks	49.6 ± 2.3 a
<i>K. streptantha</i>	3 days	4 weeks	–	

¹*K. b.*: *K. blossfeldiana*

²Time after protoplast isolation

³The culture did not reach this stage

The number of calli bigger than 1 mm was counted after 8 weeks of culture; values represent mean \pm SD, $n=3$; values followed by different letters are significantly different ($p \leq 0.05$) according to Tukey’s honestly significant difference test

was obtained in media supplemented with 0.5 mg/l BAP (Fig. 4a and b). The results of two-way ANOVA (Table 5) showed that the BAP concentration had a significant influence on the calli number. Moreover, the interaction between the effect of BAP and the effect of 2,4-D was statistically significant. The effect of 2,4-D was significant only in repetition 1 (Table 5).

The *K. blossfeldiana* ‘Paris’ protoplast derived calli number differed between repetition 1 and 2. In repetition 1, the lowest calli numbers were obtained in media supplemented with 0.05 mg/l BAP. Repetition 2 showed higher calli numbers. The results of two-way ANOVA revealed that the concentration of BAP had significant influence on the calli numbers in both repetitions. In repetition 1, there was a significant interaction between the effect of BAP and 2,4-D on the calli number while 2,4-D had no significant effect. In repetition 2 however, 2,4-D had significant influence on the calli numbers, but no interaction was observed between the effect of BAP and 2,4-D (Table 5).

For plant regeneration, all calli were transferred to solid medium containing the same PGR as in the initial cultures. The total and average numbers of calli as well as the percentage of green and yellow calli (Fig. 1e and f) after 8 and 13 weeks of culture are shown in Supplementary Table 1 and 2. After 13 weeks of culture, the calli reached 0.5–0.8 cm in diameter. The biggest (at least 7 mm) calli were cut and all calli were divided between two media supplemented with

either NAA and BAP, or NAA and zeatin (Supplementary Table 3 and 4). After 17 weeks of culture of *K. blossfeldiana* ‘Charming Red Meadow’, roots were regenerated on the medium supplemented with NAA and zeatin, from initial PGR treatment F (0.1 mg/l NAA, 0.1 mg/l BAP and 0.1 mg/l 2,4-D) in repetition 1 and 2, and from treatment L (0.1 mg/l NAA, 0.5 mg/l BAP and 1.0 mg/l 2,4-D) in repetition 2 (Table 6). These roots failed to regenerate into plants after further transfer. Shoots (Fig. 1g) were obtained from green calli of *K. blossfeldiana* ‘Charming Red Meadow’ on the medium containing NAA and BAP from initial PGR treatment F (0.1 mg/l NAA, 0.1 mg/l BAP and 0.1 mg/l 2,4-D) in repetition 1, and treatment L (0.1 mg/l NAA, 0.5 mg/l BAP and 1.0 mg/l 2,4-D) in repetition 2 (Table 6). All shoots successfully developed into plants (Fig. 1h). The callus regeneration from *K. blossfeldiana* ‘Paris’ yielded shoots only in repetition 2 on medium containing NAA and BAP from initial treatment L (0.1 mg/l NAA, 0.5 mg/l BAP and 1.0 mg/l 2,4-D) (Table 6). The plants successfully developed roots on the medium supplemented with IAA.

Discussion

Constant development of new decorative cultivars is a prerequisite for breeders and producers to be competitive within floriculture. Interspecific hybrids are important breeding material that can provide novel traits for commercial ornamental plants (Kuligowska et al. 2016). They can be obtained through sexual hybridization or somatic hybridization. The second method provides tools to avoid hybridization barriers related to sexual reproduction and can allow development of hybrids from more distantly related species (Eeckhaut et al. 2013; Kuligowska et al. 2016). The ability to isolate high numbers of healthy protoplasts enables a successful protoplast culture and development of an efficient protoplast-to-plant system (Rahmani et al. 2016). However, its establishment is influenced by many factors, among others: genotype, physiological status and growing conditions of the protoplast source tissue, protoplast isolation and medium composition (Davey et al. 2005; Eeckhaut et al. 2013).

In the present study, we successfully isolated protoplasts from 12 *Kalanchoë* accessions. A previous study of protoplast cultures in *Kalanchoë* reported a maximal yield of 6.0×10^5 protoplasts per gfw from *K. blossfeldiana* ‘Tenorio’ (Castelblanque et al. 2010). However, our preliminary studies did not produce satisfactory results following the described isolation protocol (data not shown). Using a newly developed isolation protocol based on Duquenne et al. (2007), four out of 12 *Kalanchoë* accessions showed a yield higher than 7.0×10^5 protoplasts per gfw, with the highest yield of $10.78 \pm 0.51 \times 10^5$ protoplasts per gfw obtained

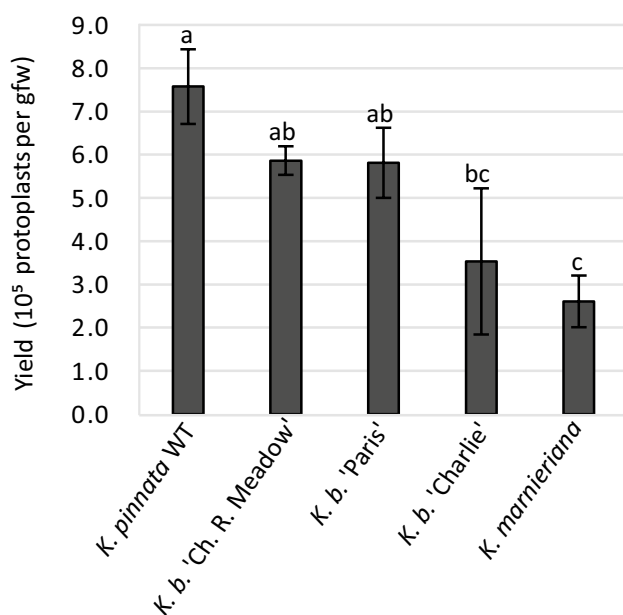


Fig. 3 Protoplast yield in experiment 2; bars represent mean \pm SD, $n=3$; bars followed by different letters are significantly different ($p \leq 0.05$) according to Tukey's honestly significant difference test; gfw: gram fresh weight; *K. b.*: *K. blossfeldiana*; 'Ch. R. Meadow': 'Charming Red Meadow'

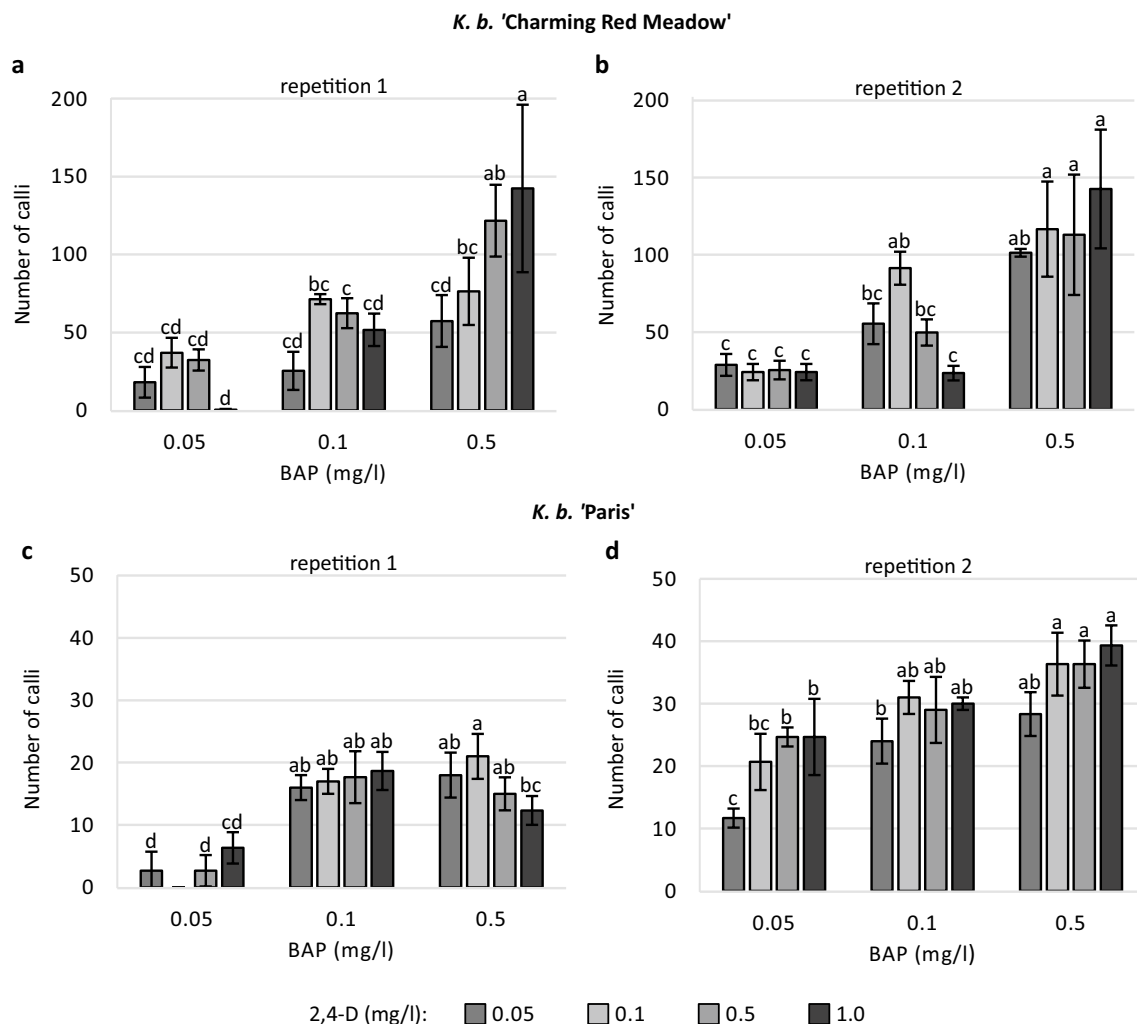


Fig. 4 Number of calli after 8 weeks according to the PGR concentrations in experiment 2 **a** and **b** repetition 1 and 2 for *K. blossfeldiana* 'Charming Red Meadow'; **c** and **d** repetition 1 and 2 for *K. blossfeldiana*

ana 'Paris'; bars represent mean \pm SD, $n=3$; bars followed by different letters are significantly different ($p \leq 0.05$) according to Tukey's honestly significant difference test

Table 5 ANOVA results (p values) of the effect of BAP, 2,4-D and BAP \times 2,4-D interaction on calli numbers in experiment 2

	<i>K. b. 'Charming Red Meadow'</i>		<i>K. b. 'Paris'</i>	
	Repetition 1	Repetition 2	Repetition 1	Repetition 2
BAP	$p < 0.001^{***}$	$p < 0.001^{***}$	$p < 0.001^{***}$	$p < 0.001^{***}$
2,4-D	$p = 0.002^{**}$	$p = 0.292$	$p = 0.920$	$p < 0.001^{***}$
BAP \times 2,4-D	$p = 0.002^{**}$	$p = 0.009^{**}$	$p = 0.006^{**}$	$p = 0.547$

K. b.: *Kalanchoë blossfeldiana*

$n=3$, $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$

in *K. blossfeldiana* 'Charming Red Meadow'. Compared to Castelblanque et al. (2010) we were able to increase the duration of enzymatic digestion from 4 h to overnight by

increasing the osmotic pressure of enzyme solution (change of mannitol concentration) and modifying enzyme concentrations. Despite a great increase of protoplast yield in some of the tested accessions, several plant accessions yielded significantly less protoplasts compared to the protoplast yield obtained by Castelblanque. Similar to our findings, Maćkowska et al. (2014) found that commercial plants exhibit higher protoplast yields than wild species. A plausible reason for differences in protoplast yields in our study is the leaf morphology of the tested species. High protoplast yields were generally obtained from plants with large, flat and thin leaves (*K. blossfeldiana*, *K. pinnata*). The low protoplast yields were observed in plants with small curly leaves or leaves covered with hair or wax (*K. aromatica*, *K. pumila*, *K. rotundifolia*). Moreover, the low protoplast yield was due to incomplete tissue digestion, suggesting that leaf morphology, at least partly disabled leaf tissue penetration

Table 6 Number of cultured calli and regenerated organs in experiment 2 after 17 weeks of culture

Initial treatment	Regeneration treatment			
	NAA (1 mg/l) + zeatin (2 mg/l)		NAA (1 mg/l) + BAP (2 mg/l)	
	Repetition 1 ^a	Repetition 2	Repetition 1	Repetition 2
<i>K. blossfeldiana</i> ‘Charming Red Meadow’				
A	0	1 (0+1)	1 (1+0)	1(1+0)
B	42 (6+36)	6 (3+3)	29 (12+17)	5(1+4)
C	4 (0+4)	7 (0+7)	2 (0+2)	11(0+11)
D	0	17 (8+9)	0	26(14+12)
E	31 (3+28)	7 (0+7)	20 (10+10)	8(7+1)
F	110 (24+86)	1 root 51 (23+28)	17 roots 104 (75+29)	4 shoots 47(33+14)
G	35 (10+25)	36 (13+23)	40 (23+17)	28(12+16)
H	35 (9+26)	28 (16+12)	41 (24+17)	26(22+4)
I	23 (8+15)	63 (36+27)	34 (27+7)	78(63+15)
J	44 (36+8)	80 (44+36)	45 (36+9)	91(61+30)
K	63 (25+38)	125 (58+67)	73 (54+16)	129(95+34)
L	44 (31+13)	150 (82+68)	5 roots 51 (33+18)	151(110+41) 8 shoots
<i>K. blossfeldiana</i> ‘Paris’				
A	0	0	0	0
B	0	1 (0+1)	0	7(0+7)
C	0	5 (0+5)	0	8(2+6)
D	0	14 (4+10)	0	14(10+4)
E	2 (0+2)	13 (0+13)	2 (0+2)	21(17+4)
F	5 (0+5)	7 (0+7)	12 (8+4)	17(8+9)
G	9 (0+9)	17 (1+16)	11 (5+6)	13(4+9)
H	10 (3+7)	23 (4+19)	16 (10+6)	26(16+10)
I	13 (2+11)	24 (1+23)	17 (12+5)	24(6+18)
J	16 (1+15)	31 (2+29)	13 (4+9)	25(11+14)
K	11 (3+8)	36 (4+32)	17 (14+3)	40(10+30)
L	8 (3+5)	32 (5+27)	8 (7+1)	40(14+16) 2 shoots

NAA 1-naphthaleneacetic acid, BAP 6-benzylaminopurine

^aTotal number of calli (green calli + yellow calli)

by the enzymes. This assumption can be supported by the lack of differences in protoplast viability among different plant accessions. Decreased protoplast yield observed in experiment 2 (started in December 2017) compared to values obtained in experiment 1 (started in September 2017) can be attributed to seasonal variation (Keskitalo 2001; Maćkowska et al. 2014). The physiological status of the source tissue is important for successful protoplast isolation (Davey et al. 2005; Lord and Gunawardena 2010). In the present study, plants with fast in vitro growth generally yielded more protoplasts. Plants between 4 and 6 weeks were suitable for protoplast isolation. In contrast, a previous study with *K. blossfeldiana* ‘Tenorio’ used 8-week-old plants for protoplast isolation (Castelblanque et al. 2010).

Development of an efficient protoplast regeneration method relies on multiple parameters related to the culture conditions in several consecutive steps. Optimization of in vitro culture requires empirical testing of different

parameters such as media composition, PGR supplementation or cultivation conditions (Davey et al. 2005). In the present study, we used medium according to Kao and Michayluk (1975) for protoplast culture. In a preliminary study using *K. blossfeldiana* ‘Charming Red Meadow’ and *K. pinnata* WT we compared KAO medium and MS-based medium according to Castelblanque et al. (2010). We only observed protoplast divisions using the first medium (data not shown). Auxins and cytokinins are the major growth regulators of sustained protoplast growth (Davey et al. 2005). In experiment 1, we used the PGR supplementation described by Castelblanque et al. (2010). The protoplast proliferation and regeneration showed genotype specific differences, as previously observed in other plant species (Meyer et al. 2009; Maćkowska et al. 2014; Bruznican et al. 2017). Even though all tested plants underwent the first protoplast divisions and we observed microcolony and even microcallus formation in some accessions, we were not able to regenerate

plants from the obtained callus. We hypothesized that the relatively high auxin concentration can contribute to callus recalcitrancy. Thus, in experiment 2 the NAA concentration was lowered to 0.1 mg/l, and we tested wider ranges of 2,4-D (from 0.05 to 1.0 mg/l) and BAP (from 0.05 to 0.5 mg/l) concentrations. Compared to experiment 1, in experiment 2 we generally obtained more calli for both *K. blossfeldiana* ‘Charming Red Meadow’ and ‘Paris’. These results indicate that a lower NAA concentration had a positive effect on callus formation in *K. blossfeldiana*. However, in *K. pinnata* WT, calli were obtained only in experiment 1. Thus, it is likely that this species requires high NAA concentration for callus formation. In the experiment 2, we observed the differences in number of calli between two repetitions. However, we observed highly significant BAP effect on calli yield in both repetitions, while 2,4-D effect and PGR interaction effect were not consistent. This can be due to the above mentioned physiological status of the source tissue. Moreover, it is likely that higher number of technical repeats are needed for clear assessment of PGR effects.

For plant regeneration from protoplast-derived callus, we used media supplemented with combinations of either NAA and BAP, or NAA and zeatin. NAA and BAP induce shoot regeneration from protoplast cultures of *K. blossfeldiana* (Castelblanque et al. 2010) and *Robinia pseudoacacia* (Kanwar et al. 2009). Zeatin is frequently combined with NAA and used in protoplast regeneration protocols for shoot induction e.g. in *Solanum* (Borgato et al. 2007) and *Albizia julibrissin* (Rahmani et al. 2016). Moreover, zeatin has a positive effect on shoot regeneration from leaf-derived callus of *K. blossfeldiana* (Varga et al. 1988). However, in the present study contrary to the expected results of the shoot development, medium supplemented with zeatin promoted root development in *K. blossfeldiana* ‘Charming Red Meadow’. The promotive effect of zeatin on root development was previously described in *in vitro* cultures of *Vaccinium vitis-idaea* and *Fragaria ananassa* (Debnath 2005, 2006). NAA and BAP were able to induce healthy shoots from both *K. blossfeldiana* cultivars and further plants were regenerated.

In the future research further efforts are needed to accomplish the increase in the shoot regeneration efficiency. The optimal medium supplementation with PGRs for shoot regeneration from protoplast-derived callus varies depending on the plant species and even genotypes (Davey et al. 2005; Rahmani et al. 2016). Sanikhani et al. (2006) and Lütken et al. (2011) reported successful shoot regeneration in *Kalanchoë blossfeldiana* by TDZ. Furthermore, shoot differentiation was induced from protoplast-derived callus in the presence TDZ in *Ulmus americana* (Jones et al. 2015). Thus, testing a wider range of PGRs including TDZ for shoot induction in *Kalanchoë* should be considered. Previously reported protoplast-to-plant system for *Kalanchoë* mesophyll

protoplasts (Castelblanque et al. 2010) as well as this study describe plant regeneration only via shoot organogenesis. Chao et al. (2004) described somatic embryogenesis induction in *K. blossfeldiana* on MS medium supplemented with NAA, BAP and activated carbon. Development of efficient regeneration system through indirect somatic embryogenesis could provide an alternative route to obtain high shoot regeneration efficiency in *Kalanchoë*. Sucrose is a regular choice of carbon source in the most protoplast cultivation media (Davey et al. 2005). However, a change in the carbon source from sucrose to glucose or maltose could promote shoot regeneration for protoplast-derived cells as demonstrated in case of *Lilium* and rice (Godo et al. 1996; Jain et al. 1995).

In conclusion, our work presents an efficient protocol of protoplast isolation from fast growing *Kalanchoë* species. The results demonstrate that protoplast yield, but not viability can be influenced by the genotype of donor plant and their growth *in vitro*. The PGR concentration used during protoplast culture is critical for callus development. However, the efficiency of colony and microcalli formation is strongly genotype-dependent. Despite the high number of obtained calli, the shoot regeneration rate was low. Further research is needed to optimize this final step of a protoplast-to-plant regeneration system in *Kalanchoë*.

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Authors contributions JC, KKM, TE, RM, HL: study conception and design; JC: Acquisition of data; JC, KKM: Analysis and interpretation of data; JC, KKM: Drafting of manuscript; JC, KKM, TE, RM, HL: Critical revisions.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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